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Abstract: Glutamine synthetase (GS) deficiency is an ultra-rare inborn error of amino acid metabolism that has been described in only three patients so far. The disease is characterized by neonatal onset of severe encephalopathy, low levels of glutamine in blood and cerebrospinal fluid, chronic moderate hyperammonemia, and an overall poor prognosis in the absence of an effective treatment. Recently, enteral glutamine supplementation was shown to be a safe and effective therapy for this disease but there are no data available on the long-term effects of this intervention. The amino acid glutamine, severely lacking in this disorder, is central to many metabolic pathways in the human organism and is involved in the synthesis of nicotinamide adenine dinucleotide (NAD(+)) starting from tryptophan or niacin as nicotinate, but not nicotinamide. Using fibroblasts, leukocytes, and immortalized peripheral blood stem cells (PBSC) from a patient carrying a GLUL gene point mutation associated with impaired GS activity, we tested whether glutamine deficiency in this patient results in NAD(+) depletion and whether it can be rescued by supplementation with glutamine, nicotinamide or nicotinate. The present study shows that congenital GS deficiency is associated with NAD(+) depletion in fibroblasts, leukocytes and PBSC, which may contribute to the severe clinical phenotype of the disease. Furthermore, it shows that NAD(+) depletion can be rescued by nicotinamide supplementation in fibroblasts and leukocytes, which may open up potential therapeutic options for the treatment of this disorder.

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ORIGINAL ARTICLE

Secondary NAD^+ deficiency in the inherited defect of glutamine synthetase

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Abstract Glutamine synthetase (GS) deficiency is an ultra-rare inborn error of amino acid metabolism that has been described in only three patients so far. The disease is characterized by neonatal onset of severe encephalopathy, low levels of glutamine in blood and cerebrospinal fluid, chronic moderate hyperammonemia, and an overall poor prognosis in the absence of an effective treatment. Recently, enteral glutamine supplementation was shown to be a safe and effective therapy for this disease but there are no data available on the long-term effects of this intervention. The amino acid glutamine, severely lacking in this disorder, is central to many metabolic pathways in the human organism and is involved in the synthesis of nicotinamide adenine dinucleotide (NAD^+) starting from tryptophan or niacin as nicotinate, but not nicotinamide. Using fibroblasts, leukocytes, and immortalized peripheral blood stem cells (PBSC) from a patient carrying a *GLUL* gene point mutation associated with impaired GS activity, we tested whether glutamine deficiency in this patient results in NAD^+ depletion and whether it can be rescued by supplementation with glutamine, nicotinamide or nicotinate. The present study shows that congenital GS deficiency is associated with NAD^+ depletion in fibroblasts, leukocytes and PBSC, which may contribute to the severe clinical phenotype of the disease. Furthermore, it shows that NAD^+ depletion can be rescued by nicotinamide supplementation in fibroblasts and leukocytes, which may open up potential therapeutic options for the treatment of this disorder.

Abbreviations

GS	Glutamine synthetase
NAD^+	Nicotinamide adenine dinucleotide
PBSC	Peripheral blood stem cells

Introduction

Glutamine is an abundant, non-essential amino acid and is central for a wide range of metabolic reactions in all mamma-

Dedication The authors want to dedicate this work to the late Hamad Ajool, the then only known patient with inherited GS deficiency, who was able to give so much to his family and to science.

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lian organisms (Häussinger 1998). The main functions of glutamine include storage and transport of nitrogen between different organs during fetal development as well as later in life. The liver, together with skeletal muscle, is the central organ for maintaining glutamine homeostasis by removing or releasing glutamine from or into the circulation, respectively, as required. In the central nervous system, astroglial cells are responsible for maintaining glutamine homeostasis in the cerebrospinal fluid (Chaudhry et al 1999). Glutamine is synthesized in the human body solely by the reaction of glutamine synthetase (GS; synonymous: glutamate-ammonia ligase; EC 6.3.1.2), a key enzyme in nitrogen metabolism, requiring glutamate, ammonia, and ATP (Farrow et al 1990; Häussinger 1990, 1998). The rate of glutamine synthesis depends on substrate availability, and the expression level and activity of GS (Häberle et al 2006a). L-glutamine, not D-glutamine, is the only substrate of the γ-glutamyl transfer reaction of GS (Wu 1977).

GS is predominantly expressed in brain, kidney, muscle, and liver (Häussinger and Sies 1984; He et al 2010; Krajewski et al 2008; Liaw et al 1995). In humans, several studies into the role of GS have focused on the brain (Derouiche and Ohm 1994; Tumani et al 1995; Yamamoto et al 1987) to better understand various conditions including epilepsy (Eid et al 2004), schizophrenia, Parkinson's disease (Carlsson and Carlsson 1990), Huntington's chorea (Young et al 1988), Alzheimer's disease (Hardy and Cowburn 1987) and hepatic encephalopathy (Häussinger et al 1994). GS is effectively and irreversibly inhibited by methionine sulfoximine (Abramson et al 1991).

Inherited GS deficiency (MIM #610015) due to a defect in the *GLUL* gene (MIM *138290) is an ultra-rare disorder described in only three patients who all suffered from neonatal onset severe epileptic encephalopathy. The pathophysiology of GS deficiency is thought to be related to the indispensable role of GS as the only enzyme for glutamine synthesis in particular since glutamine is the unique amino moiety donor for many essential metabolites including purines and pyrimidines, amino acids, glucose precursors, adenosine monophosphate, and nicotinamide adenine dinucleotide (NAD^+) (Häberle et al 2006b). Treatment of GS deficiency was suggested with regular enteral glutamine supplementation but this concept could only be tested in a single patient so far (Häberle et al 2012).

Nicotinic acid, nicotinamide, or nicotinamide riboside are the precursors for generation of NAD^+ (Fig. 1). These are collectively and colloquially referred to as niacin or vitamin B3. The unphosphorylated and phosphorylated forms, NAD^+/NADH and NADP/NADPH, respectively, are essential for numerous oxido-reduction reactions in the cell (Bieganowski et al 2003). The transfer of electrons from NADH to oxygen is essential for adenosine triphosphate (ATP) formation in aerobic metabolism. NAD^+ can modulate several cellular

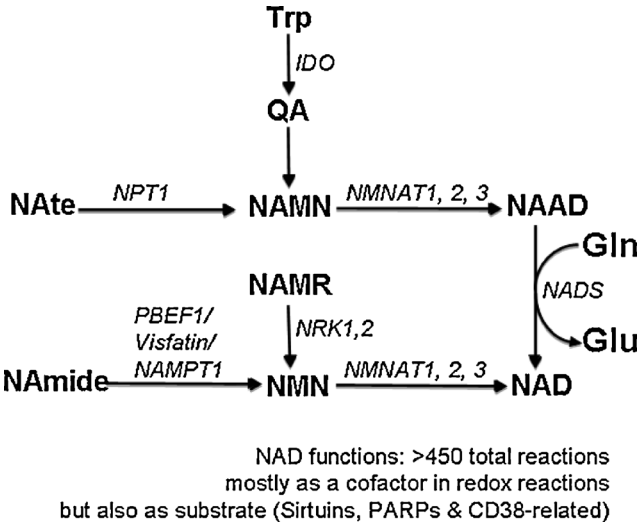

Fig. 1 Biochemical pathways involved in the synthesis of NAD^+. Scheme of NAD^+ synthesis from deamino-NAD^+ requiring the amino group from glutamine. Included are also alternative routes for NAD^+ generation starting from nicotinate, nicotinamide, nicotinamide riboside, or tryptophan. Respective genes are denoted in italic. Abbreviations: *Gln* glutamine, *Glu* glutamate; *IDO* indolamin-2,3-dioxygenase, *NA* nicotinate/niacinate/nicotinic acid, *NaAD* nicotinic acid adenine dinucleotide, *NAD* nicotinamide adenine dinucleotide, *NADS* NAD synthetase, *NAM* nicotinamide, *NaMN* nicotinic acid mononucleotide, *NAMPT1* nicotinamide phosphoribosyltransferase (synonymous to *Visfatin* or *PBEF1*, pre-B-cell colony-enhancing factor 1), *NAMR* nicotinamide riboside, *NMN* nicotinamide mononucleotide, *NMNAT1,2,3* nicotinamide mononucleotide adenylyltransferase 1,2,3, *NPT1* nicotinate phosphoribosyltransferase, *NRK1,2* Nicotinamide Riboside Kinase 1,2, *PARPs* poly (ADP-ribose) polymerase family, *Trp* tryptophan, *QA* quinolinic acid

functions such as sensitivity of cells to apoptosis (Luo et al 2001), but the mechanisms regulating cellular NAD^+ through metabolic events remain to be fully elucidated. In humans, NAD^+ is synthesized from deamino-NAD^+, ATP, and an amino group from glutamine, a reaction catalyzed by NAD^+ synthetase (Fig. 1). In addition to the glutamine-dependent NAD^+ synthetase in mammalian cells, there exists an ammonia-dependent NAD^+ synthetase in prokaryotes with different amide donor specificity and tissue distributions (Hara et al 2003; Spencer and Preiss 1967).

Previously, we had reported on the only living of hitherto three known GS deficient patients who suffered from chronic moderate hyperammonemia and severe neonatal–onset encephalopathy (Häberle et al 2011). In this study, we test the hypothesis that systemic glutamine deficiency impairs NAD^+ synthesis, and that NAD^+ deficiency can be rescued by supplementation with nicotinamide or nicotinic acid. We therefore studied NAD^+ levels in different cell lines derived from the only living GS deficient patient, and indeed found NAD^+ deficiency. To our knowledge, this is the first description of NAD^+ deficiency in a patient with systemic glutamine deficiency caused by a mutation in the *GLUL* gene. Given the importance of NAD^+, the severe phenotype in GS deficiency

may well be caused by the combined deficiencies of glutamine and of NAD^+. This finding adds to our understanding of the pathophysiology of GS deficiency and supports that nicotinamide supplementation may be beneficial for treating NAD^+ deficiency in this particular disorder.

Material and methods

Culture of fibroblasts

Patient and control (n=3) fibroblasts were cultured in T75 flasks containing 8 ml of standard DMEM containing 4 mM GlutaMAX (Invitrogen, Basel, Switzerland), 10 % FBS (PAA Laboratories, GE Healthcare, Glattbrugg, Switzerland) and antibiotic-antimycotic (Forget et al 1999). Forty eight hours prior to cell harvest, the medium was aspirated and cells were washed with DPBS (Invitrogen). Then, cells were grown for 48 h in 8 ml of above medium without glutamine or supplemented with 10 mM GlutaMAX (Gln) (Invitrogen), 1 mM nicotinamide (NAmide) (Sigma-Aldrich, Buchs, Switzerland), 1 mM nicotinic acid (anionic form: nicotinate) (NAte) (Sigma-Aldrich) or with a combination of 10 mM glutamine and 1 mM nicotinamide.

Fibroblast harvest and cell count

Fibroblasts were washed with PBS and trypsinized for 10 min at 37 °C with 1 ml 0.25 % trypsin (Invitrogen) before 4 ml DMEM was added to the cells. Suspension of cells was kept as aliquots of 800 μl in Eppendorf tubes. After centrifugation for five min at 500 g, the supernatant was discarded and pellets, immediately frozen in liquid nitrogen, were stored at -80 °C until NAD^+ measurement. To count viable cell numbers using an improved Neubauer chamber (Brand GmbH, Wertheim, Germany), another 100 μl of the cell suspension was mixed with 100 μl of trypan blue (Sigma-Aldrich).

Leukocytes harvest and cell count

To study the safety and efficacy of glutamine supplementation, a GS deficient patient was treated for 4 weeks with L-glutamine (doses increasing to a maximum of 1020 mg/kg bodyweight/day) (Häberle et al 2012). Before treatment and after the maximum dose of glutamine was achieved, 10 ml of heparin blood and 1 ml of EDTA blood were collected from the patient and from untreated controls (n=3). Leukocytes were counted in EDTA blood by using an automated haematology analyzer (Sysmex AG, Horgen, Switzerland). Heparin blood was used to extract leukocytes. For this, red blood cells were lysed in 30 ml lysis buffer (10 mM $KHCO_3$ and 155 mM NH_4Cl) for 15 min prior to centrifugation at 500 g for 5 min. Washing of the leukocyte pellet was done with decreasing concentrations of NaCl (0.68, 0.34, and 0.17 %) and centrifugation at 850 g for 5 min that was repeated three times. Cells in 0.17 % NaCl solution were aliquoted to 6 million leukocytes per vial following the centrifugation for 5 min at 500 g, the supernatant discarded and pellets immediately frozen in liquid nitrogen until NAD^+ measurement.

Transformation of B-lymphocytes to establish peripheral blood stem cells (PBSC)

Peripheral blood B-cells were transformed using wild-type EBV B95.8 as described earlier (Dorner et al 2008). Briefly, peripheral blood mononuclear cells (PBMC) from the patient, his father and from three controls were isolated by Ficoll gradient centrifugation. Nucleated red blood cells in the patient sample were lysed using lysis buffer (10 mM $KHCO_3$ and 155 mM NH_4Cl) for 15 min. Unseparated PBMC were infected with supernatant from a 4-day culture of EBV B95.8 by conventional inoculation. Cells were cultured in 96-well plates containing RPMI 1640 (Invitrogen) with 10 % FBS (PAA), antibiotic-antimycotic (PAA) and 2 mM GlutaMAX (Invitrogen). One half of the RPMI 1640 medium was changed every 3 days for 4 weeks.

Growth of peripheral blood stem cells

Peripheral blood stem cells (PBSC) were cultured in upright T25 flasks containing 20 ml RPMI 1640 (Invitrogen) containing 10 % FBS (PAA), antibiotic-antimycotic (PAA) and 2 mM GlutaMAX (Invitrogen) up to a cell density of at least 300,000 cells per ml. Aggregated cells were dissociated twice weekly by gentle trituration with a pipette. To split cells or to change medium, cells were centrifuged for 5 min at 100 g and resuspended in fresh medium. For glutamine and nicotinamide experiments, cells were grown in the same medium but without or with GlutaMAX (10 mM) or 1 mM nicotinamide for 72 h. To count cells, these were dissociated and 100 μl of the cell suspension was mixed with 100 μl of trypan blue (Sigma-Aldrich) and counted using an improved Neubauer chamber (Brand). The cell solution was aliquoted in order to get 6 million PBSC per vial. These vials were then centrifuged for 5 min at 500 g, the supernatant discarded and the pellets immediately frozen in liquid nitrogen until NAD^+ measurement.

Protein extraction and Western blot analysis

Fibroblasts derived from patient and controls were treated with or without 10 mM glutamine and/or 1 mM nicotinamide for 2 or 3 days. Media were changed daily. Cells were harvested and lysed in complete Nonidet P-40 (Roche Applied Science, Rotkreuz, Switzerland) lysis buffer containing 1 % Nonidet P-40, 50 mM Tris–HCl (pH 8), 125 mM NaCl, 1 mM EDTA, and protease inhibitors (1x Complete EDTA-free+

1 mM of PMSF) (Roche Applied Science). Cell lysates were then centrifuged at 4 °C for 15 min at 27,000 g. Protein concentrations were determined by the method of Lowry (Lowry et al 1951) using bovine serum albumin (BSA) as standard.

Western blotting was performed as described previously (Laemmli 1970). A total of 135 μg protein (except for experiments shown in Fig. 2 where 5–40 μg were used as indicated) was separated by 10 % denaturing SDS-PAGE and subsequently transferred to nitrocellulose transfer membranes (Whatman GmbH, Dassel, Germany). The primary polyclonal antibody anti-GS (Sigma-Aldrich, G2781), recognizing GS amino acids 357–373 with N-terminally added lysine according to the manufacturer, was used at a dilution of 1:1000, and the horseradish peroxidase (HRP)-conjugated secondary antibody anti-rabbit (Santa Cruz Biotechnology, Inc., Heidelberg, Germany) was used at a dilution of 1:7500. Antibody against β-actin (Santa Cruz Biotechnology) served as loading control. Protein detection was done using ECL reagents (GE Healthcare, Little Chalfont, UK) for chemiluminescent labeling.

NAD^+ measurement

NAD^+ was measured using the EnzyChrom NAD^+/NADH Assay Kit (BioAssay Systems, Lausanne, Switzerland) according to the manufacturer's protocol. This assay is highly specific for NAD^+ with no interference from $NADP^+$. To make sure that samples were comparable, only samples with 350,000 to 550,000 cells in a fixed volume of 800 μl were analyzed. In brief, cell-pellets were suspended in 100 μl of NAD^+ extraction buffer and lysed using a small douncer and pestle. Cell extracts were heated at 60 °C for 5 min and then 20 μl assay buffer was added as well as 100 μl of the opposite extraction buffer to neutralize the extracts. The samples were briefly vortexed and spun down at 14,000 rpm for 5 min. Of the supernatant, 40 μl was loaded with 80 μl working reagent into a clear flat-bottom Greiner UV-Star 96-well plate (Sigma) for NAD^+ assay. The optical density was measured for time "zero" and after 15-min incubation at 37 °C at 565 nm using Infinite M200 plate-reader and the i-control6 software (both from Tecan, Männedorf, Switzerland). NAD^+ levels are given per number of cells in the assay and presented as [nmol $NAD^+/10^5$ cells].

Statistics

Statistical analyses were done by one-way analysis of variance (ANOVA) with the program GraphPad Prism 4 (GraphPad Software, San Diego, CA) to describe the differences of NAD^+ between controls' and patient's fibroblasts, leukocytes, and lymphocytes. Standard deviations (SD) are shown in Table 1. Differences were considered statistically significant if p-value was <0.05.

Results

Upregulated GS expression in patient cells and in absence of glutamine

To determine the level of GS expression in cultured human fibroblasts, we performed Western blotting (Fig. 2a). GS

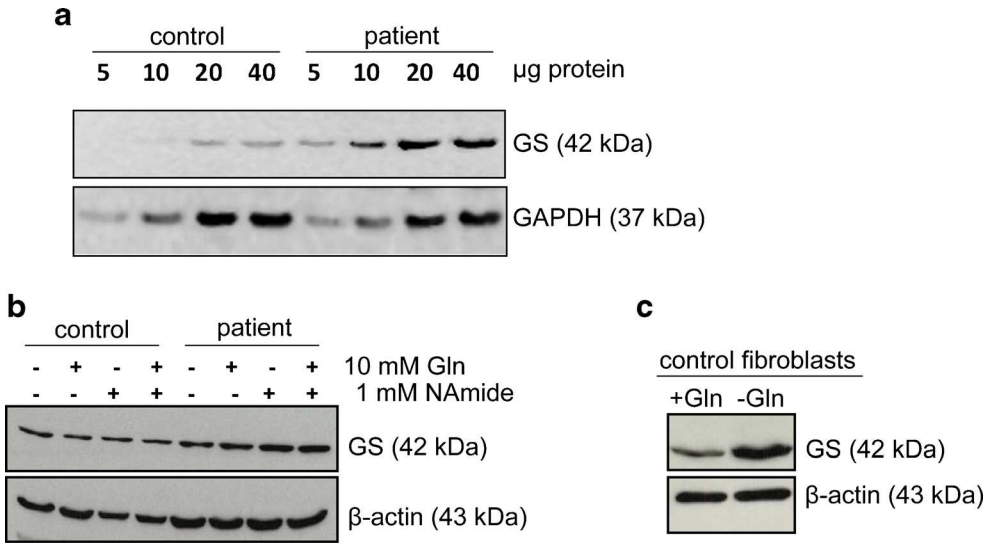

Fig. 2 GS expression in cultured human fibroblasts of a control or a patient with glutamine deficiency. **a**: Analysis of GS protein expression in cultured fibroblasts with indicated amounts (5, 10, 20, 40 μg, respectively) by Western-blot analysis. **b**: Effect of glutamine (Gln) and nicotinamide (NAmide) on GS protein expression. Cells were cultured in the presence (+) or absence (-) of glutamine (Gln) or nicotinamide (NAmide) for 48 h. **c**: GS protein expression in control fibroblasts cultured for 72 h in presence (+Gln) or absence (-Gln) of glutamine (4 mM). GAPDH (**a**) or β-actin (**b**, **c**) served as a loading control

Table 1 NAD^+ in fibroblasts, leukocytes, and peripheral blood stem cells (PBSC) from the patient, patient's father, and controls

Samples	Patient					Patient's father		Controls			
	-Gln	+Gln	+NAmide	+NAte	+Gln + Namide	-Gln	+Gln	-Gln	+Gln	+NAmide	+NAte
NAD^+ (nmol/10^5 fibroblasts) ± S.D.	0.14±0.07	**0.36±0.19**	**0.47±0.22**	**0.50±0.09**	**0.50±0.08**	N.D.	N.D.	0.41±0.17	0.46±0.21	0.46±0.25	0.47±0.27
NAD^+ (nmol/10^5 leukocytes) ± S.D.	0.01±0.01	**1.31±0.40**	N.D.	N.D.	N.D.	N.D.	N.D.	0.45±0.21	N.D.	N.D.	N.D.
NAD^+ (nmol/10^5 PBSC) ± S.D.	0.07±0.02	0.05±0.03	0.04±0.03	N.D.	N.D.	0.12±0.01	0.08±0.02	0.13±0.03	0.12±0.03	N.D.	N.D.

S.D., standard deviation; N.D., not determined

In bold: levels significantly different from untreated situation (-Gln)

protein was markedly upregulated in fibroblasts from the patient with glutamine deficiency as compared to human fibroblasts of a control. To study whether exogenous glutamine or nicotinamide supplementation affects GS protein expression, Western blotting was performed on homogenates from untreated cells or cells treated with glutamine or nicotinamide for 48 h (Fig. 2b) or from control cells cultured with or without glutamine for 72 h (Fig. 2c). Control cells cultured in the presence of 10 mM (Fig. 2b) or 4 mM (Fig. 2c) glutamine showed a slight decrease in GS expression compared to those cultured in the absence of glutamine. In contrast, patient cells did not show any change in the level of GS expression when grown without glutamine (Fig. 2b). Likewise, nicotinamide was without any effect on the expression levels of GS protein relative to β-actin in fibroblasts from the control as well as the patient (Fig. 2b).

Glutamine-, nicotinamide- or nicotinate- supplementation normalize NAD^+ levels in GS deficient fibroblasts

Since the reaction of NAD^+ synthetase requires glutamine, we first analyzed the NAD^+ in vitro in fibroblasts and found a significantly reduced NAD^+ level (0.14±0.07 nmol NAD^+/10^5 cells) in a patient with glutamine deficiency compared to a control (0.41±0.17 nmol NAD^+/10^5 cells) (Fig. 3, Table 1). To investigate the effect of glutamine and the NAD^+ precursors nicotinamide and nicotinate on NAD^+ synthesis in GS deficient cells, we added these agents to the culture medium of fibroblasts from patient and controls. NAD^+ in GS deficient fibroblasts treated with glutamine or NAD^+ precursors nicotinamide or nicotinate was significantly increased (0.36±0.19, 0.47±0.22 and 0.50±0.09 nmol NAD^+/10^5 cells, respectively, all significantly above the untreated cells, $p<0.01$) reaching levels comparable to controls (0.46±0.21, 0.46±0.25, and 0.47±0.27 nmol NAD^+/10^5 fibroblasts, respectively) (Fig. 3, Table 1). However, in control cells, NAD^+ levels remained unchanged under any of the agents used ($p>0.05$). Combined treatment with glutamine and nicotinamide had no synergistic effect on NAD^+ levels in GS deficient cells. These results indicate that GS deficiency results in partial NAD^+ depletion in fibroblasts, which can be rescued by supplementation with glutamine and/or? the NAD precursors nicotinamide and nicotinate.

Glutamine supplementation normalizes reduced NAD^+ levels in GS deficient leukocytes

Next, we wanted to measure the NAD^+ in vivo using leukocytes isolated from the GS deficient patient and controls. In patient's leukocytes, NAD^+ levels were close to zero (0.01± 0.01 nmol NAD^+/10^5 leukocytes; results from nine independent measurements) while control levels were similar to those

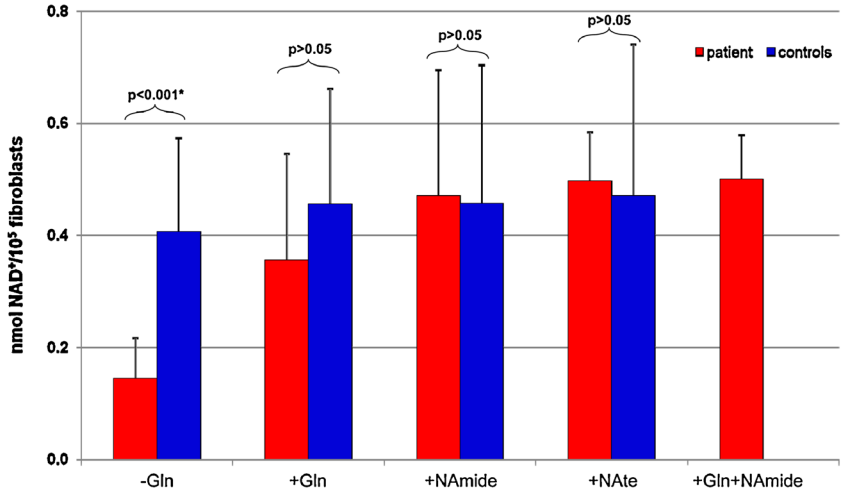

Fig. 3 NAD^+ levels in GS deficient and control fibroblasts. Control and GS deficient cultured fibroblasts were treated with 10 mM glutamine (Gln), 1 mM nicotinamide (NAmide), 1 mM nicotinate (NAte) or with a combination of 10 mM glutamine and 1 mM nicotinamide for 48 h, respectively. –Gln indicates cells were grown in standard culture medium without glutamine for 48 h. NAD^+ was measured from cell lysates using EnzyChrom NAD^+/NADH assay and presented as nmol $NAD^+/10^5$ cells. Asterisks indicate that differences were significant with a p-value<0.001

found in fibroblasts (0.45±0.21 nmol $NAD^+/10^5$ leukocytes) (Fig. 4, Table 1). This suggests that GS deficiency results in severe NAD^+ depletion in leukocytes. To test whether glutamine supplementation restores NAD^+ levels in GS deficient leukocytes, we investigated NAD^+ levels in leukocytes of the patient before and 4 weeks after treatment with L-glutamine (details of this trial in (Häberle et al 2012)). During glutamine supplementation of the patient, we observed that GS deficient leukocytes showed a marked increase in NAD^+ raising to 1.31 ±0.40 nmol $NAD^+/10^5$ leukocytes (controls 0.45±0.21 nmol $NAD^+/10^5$ leukocytes) (Fig. 4, Table 1). Taken together, these results indicate that GS deficiency is associated with NAD^+ depletion in leukocytes which can be rescued by glutamine supplementation.

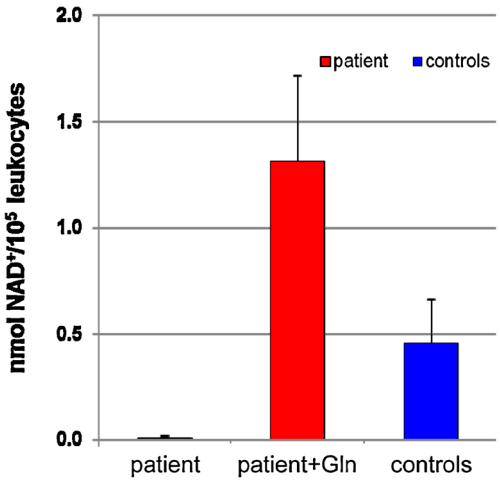

Fig. 4 NAD^+ levels in human leukocytes. Leukocytes from the GS deficient patient (obtained in vivo) before and after treatment with 10 mM glutamine (+Gln) for 4 weeks and from controls were analyzed for NAD^+ levels. NAD^+ was measured in cell lysates using EnzyChrom NAD^+/NADH assay and expressed in nmol $NAD^+/10^5$ cells

Reduced NAD^+ levels in GS deficient peripheral blood stem cells (PBSC) cannot be normalized to control levels by supplementation with glutamine or nicotinamide

In order to extend our studies to other cell types, NAD^+ level was measured in vitro using PBSC isolated from blood samples from the GS deficient patient, the patient's father, and controls. Whereas NAD^+ levels in the patient's father (0.12± 0.01 nmol $NAD^+/10^5$ PBSC) did not differ from controls (0.13±0.03 nmol $NAD^+/10^5$ PBSC), NAD^+ levels in the GS deficient PBSC (0.07±0.02 nmol $NAD^+/10^5$ PBSC) were significantly reduced when compared to PBSCs from the patient's father and controls (Fig. 5, Table 1). This indicates that GS deficiency is associated with NAD^+ depletion in PBSC.

To investigate whether glutamine- and nicotinamide-supplementation can ameliorate NAD^+ depletion in GS deficient PBSC, we treated PBSCs with L-glutamine and PBSCs from the patient additionally with nicotinamide for 72 h. Surprisingly, GS deficient PBSCs treated with L-glutamine or nicotinamide showed no change in NAD^+ levels (0.05±0.03 and 0.04±0.03 nmol $NAD^+/10^5$ PBSC, respectively) (Fig. 5, Table 1). Similarly, also PBSCs from patient's father as well as from controls treated with L-glutamine yielded unchanged NAD^+ levels. Taken together, these results indicate that GS deficiency is associated with reduced NAD^+ levels in PBSC which cannot be normalized to control levels by glutamine or nicotinamide supplementation.

Discussion

GS deficiency is an ultra-rare inborn error of metabolism with not yet fully understood pathophysiology. The most obvious cause for development of disease is the severe glutamine

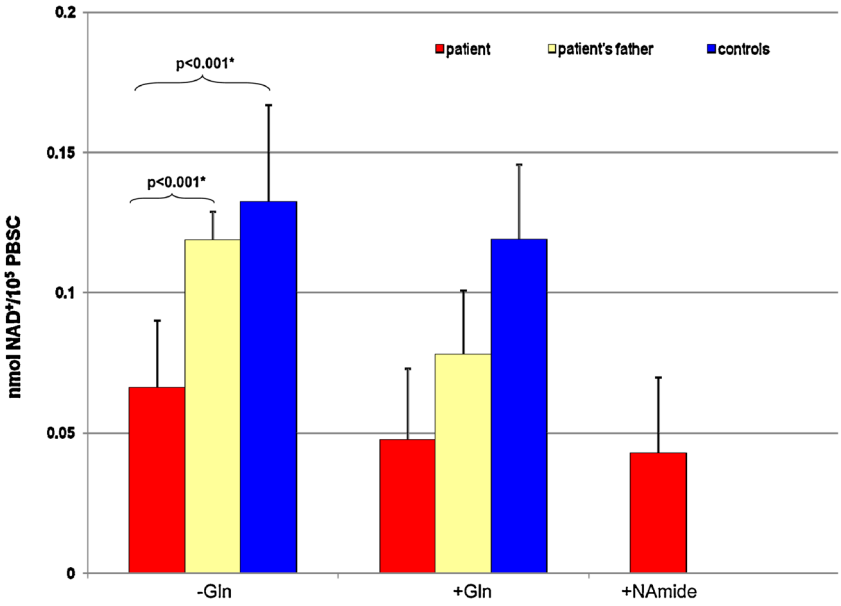

Fig. 5 NAD^+ levels in peripheral blood stem cells (PBSC). PBSC from the patient, the patient's father and controls were either left untreated or treated with glutamine ("Gln", 10 mM, 72 h) and, in case of PBSC from the patient with nicotinamide (NAmide, 1 mM, 72 h) before NAD^+ was measured in cell lysates using EnzyChrom NAD^+/NADH assay and expressed in μM $NAD^+/10^5$ cells. Asterisks indicate that differences were significant with a p-value<0.001

deficiency that was observed in all patients in different body fluids and, in one patient, in brain tissue (Häberle et al 2005, 2011). However, since glutamine plays many important roles in various cell-specific metabolic pathways in the human organism, it is expected that glutamine deficiency causes alterations in the level and function of many co-enzymes and/or co-factors which may aggravate the clinical phenotype. As such disturbances may be easily treatable and as this may have huge impact on the patients' welfare and function, it is important to identify the secondary effects of glutamine deficiency. Here we show that glutamine deficiency results in NAD^+ depletion in leukocytes, fibroblasts, and PBSC. Furthermore, we show exogenous supplementation of glutamine, nicotinamide or nicotinate rescues this biochemical phenotype in fibroblasts and in leukocytes suggesting novel therapeutics for GS deficiency, glutamine depletion, and/or NAD^+/NADH pathologies.

We took advantage of the availability of cells and cell lines from a patient with inherited GS deficiency due to the homozygous missense mutation *GLUL*-p.Arg324Ser (Häberle et al 2011). The affected residue in this mutation, arginine-324, is highly conserved and forms part of the ATP-binding site of the enzyme (Krajewski et al 2008). Moreover, arginine-324 is part of a loop, termed "the Glu327 flap" consisting of residues 323–330, that guards the glutamate entrance to the active site (Eisenberg et al 2000). We had previously performed a therapeutic trial in this patient by using enteral and parenteral glutamine supplementation that was well tolerated and resulted in an improved clinical status and partial correction of peripheral and central glutamine deficiency (Häberle et al 2012).

Fibroblasts obtained from an aborted fetus with GS deficiency previously demonstrated an upregulation of the GS protein and a significant drop in the proliferation rate (Vermeulen et al 2008). We confirm here in mature GS deficient fibroblasts the upregulation of GS supporting the use of cultured fibroblasts for experiments into the pathophysiology of the disease. When cultured in the absence of glutamine, patient cells showed a significant ($p<0.001$) NAD^+ depletion. However, this NAD^+ depletion could be rescued by addition of glutamine, nicotinamide or nicotinate to the culture medium. The combined use of glutamine and nicotinamide did not further increase the levels of NAD^+. In control cells, NAD^+ remained unchanged under any of the supplements. These findings indicate that GS protein expression is up-regulated on-demand for glutamine. Furthermore, they show that a defective GS leads to partial NAD^+ depletion in cultured fibroblasts that can be rescued by glutamine, the substrate of NAD^+ synthetase, as well as by nicotinamide or nicotinate, precursors of alternative NAD^+ generation. We measured NAD^+ in this study as the biggest measurable differences under niacin deficiency conditions, as we are exploring here with GS deficiency, can be expected for measurements of NAD^+, not NADH, NADP, or NADPH (Tang et al 2008).

In addition to the in vitro investigations in fibroblasts, we studied the situation in leukocytes in vivo. Different from fibroblasts, NAD^+ was almost absent in untreated leukocytes from the GS deficient patient while levels in controls were in the range seen in fibroblasts. NADase may have contributed to the initial result; however, as we found higher levels of NAD^+ in control patients, we exclude this to be the primary cause. The impact of glutamine for NAD^+ levels was demonstrated by marked increase in NAD^+ in leukocytes after a 4 week therapeutic trial with a final dose of glutamine of 1020 mg/kg/day (Häberle et al 2012). The increase even outnumbered the levels in non-age-matched controls. Thus, in agreement with the findings in fibroblasts, and representing strong direct clinical experimentation data, the defective GS leads to a severe NAD^+ depletion in leukocytes that can be rescued with glutamine.

To extend the studies further, we used immortalized PBSC from the GS deficient patient and his father and measured NAD^+. Similar to fibroblasts but less pronounced than in leukocytes, PBSC from the patient also showed a partial NAD^+ deficiency with levels significantly ($p<0.001$) lower than in the heterozygous father and in controls. In contrast to fibroblasts and leukocytes, levels of NAD^+ did not increase upon supplementation of glutamine or nicotinamide to the culture medium. As there are families of glutamine transporters with differential age- and cell-specific localization (Boulland et al 2003; Nissen-Meyer and Chaudhry 2013) these data suggest that PBSCs may lack proper glutamine transporters at this stage (as they are immature and undifferentiated) and thus exogenous glutamine may not contribute to stimulation of NAD^+ synthesis. Similarly, transport of nicotinamide into PBSC may have been hampered due to lack of the necessary transport systems. Alternatively, NAD^+ synthesis in PBSCs may be regulated by different mechanisms. In either case, the partial NAD^+ deficiency in PBSC cannot be rescued by exogenous supplementation of glutamine and/or nicotinamide. In conclusion, an inherited defect of GS leads, as a result of the severe lack of glutamine, to NAD^+ depletion. This appears to be cell type-specific as NAD^+ levels were significantly reduced in fibroblasts and PBSC, while NAD^+ levels in leukocytes were almost negligible. This NAD^+ depletion can be rescued in vitro in fibroblasts and in vivo in leukocytes but remains unchanged in immortalized PBSC further corroborating the cell-specific nature of glutamine- and nicotinamide transport, metabolism and action.

The finding of NAD^+ deficiency extends the pathophysiological understanding of GS deficiency and underscores the paramount importance of glutamine within the human metabolism. As a next step, it would be interesting to investigate parts of the metabolome, such as the glycolysis pathway, the citric acid cycle, anaerobic substrate level phosphorylation or ATP synthesis, that all heavily depend on sufficient NAD^+ availability. However, such studies would probably require a suitable animal model. At present, our work already opens a novel potential therapeutic option as treatment with nicotinamide, in addition to the supplementation of glutamine, it could be considered in future patients with an inherited *GLUL* defect, possibly also in secondary GS deficiency, and in patients with NAD^+ aberrations, as this may improve their brain functions and their immunity (Bruzzone et al 2009; Tullius et al 2014).

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Compliance with Ethics Guidelines

Conflict of interest None.

Human and Animal Rights and Informed Consent This article does not contain any studies with animal subjects. Studies with human material are in accordance with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from the legal guardians of the patient for being included in the study.

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